Enantiomeric Separation of Dns-DL-amino Acids by γ-Cyclodextrin-Modified Micellar Capillary Electrophoresis

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Cyclodextrin-modified micellar capillary electrophoresis (CD-MCE) was applied to enantiomeric separation of dansyl-derivatized (Dns)-DL-amino acids with sodium dodecyl sulfate (SDS). Effects of γ-CD, capillary length, organic modifiers, and applied voltage on migration time, resolution (Rs), and separation efficiency were investigated. The results show that the migration behavior of Dns-DL-amino acid is related to the cavity sizes of CDs. γ-CD has been found to be more effective in improving Rs and separation efficiency and shortening migration times, as compared to γ-CD, as a chiral selector. Increase in the length of capillary and applied voltage are found to be beneficial for increasing Rs and separation efficiency. Addition of an organic modifier, such as 10% acetonitrile, to the background electrolyte solution helped to remarkably improve the enantioselectivity and separation of D-methionine and D-leucine.

Keywords: *Enantiomeric separation; Dns-DL-amino acid; γ-cyclodextrin; micellar capillary electrophoresis (MCE)*

INTRODUCTION

The development of chiral compounds, especially in the pharmaceutical field, is placing increasing demands on analytical methods for separation of the isomers of these compounds, to achieve chiral purity control of drugs and pharmacokinetic studies. Because the enantiomers of the same drug often show different pharmacological or bioactive effects, the chiral control of drugs, pharmaceutical studies, and understanding of the drug metabolism are highly desirable and are major concerns today. Racemization and cross-linking reactions, which usually cause the exceptional formation of lysinoalanine and lanthionine, occur when the protein-containing foods are treated with alkali (Masters and Friedman, 1979; Friedman and Masters, 1982; Liardon and Hurrel, 1983). Moreover, both of these reactions happen to be the main reasons of nutritional losses. Thus, chirality analysis of the amino acids is of particular interest in the food industry. Racemization of free amino acids has been observed to be more insignificant than that of amino acids in proteins (Liardon and Ledermann, 1986), and the liability of racemization of the amino acid has been observed to be in the decreasing order aspartic acid, phenylalanine, glutamic acid, alanine, leucine, valine, and proline (Masters and Friedman, 1979).

Micellar capillary electrophoresis (MCE), based on the differential partitioning of analytes between the micelle and the surrounding aqueous phase (Terabe et al., 1989, 1993), has been reported to be effective in the separation of enantiomers (Ozaki et al., 1995). Enantiomeric separation can be achieved by using a chiral environment that interacts with the enantiomers either before or during the separation process, forming stable diastereoisomers or labile diastereomeric complex, respectively.

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Many of the chiral amino acid separations are presently carried out by MEKC with a chiral selector. Gassmann et al. (1985) have reported the enantiomeric separation of the dansylated amino acids by addition of Cu(II)/histidine complex to the electrolyte buffer solution and then using Cu(II)/aspartame as a chiral selector to elucidate the effect of temperature on resolution (Gozel et al., 1987). Guttman et al. (1988) incorporated CD into a polyacrylamide-gel-filled column to resolve the dansyl-DL-amino acids and found that γ-CD was most effective in amino acid resolution, whereas γ-CD was unsuitable for the same. The wide use of CDs and their derivatives for the resolution of enantiomers by MCE is based on their selective complexation with the analytes. Hydrophobic interactions between the analytes and the CD cavity and hydrogen bonds with hydroxyl groups on the CD rim are known to lead to the formation of labile diastereoisomeric complexes with different stability constants. The most stable complex formed moves with a lower effective mobility in the capillary during the electrophoretic operation. In addition, CDs are stable in the aqueous phase, do not absorb UV or visible light, and exhibit special chiral recognition with the analytes, thus increasing their application in enantiomeric separation (Terabe et al., 1990; Nishi et al., 1991)

Because the properties of amino acids are different, the separation of an enantiomer mixture of amino acids has to be conducted step by step according to several different methods. To establish some of the experimental conditions for enantiomeric separation of the racemic amino acids, which are formed in the alkali-treated proteins, racemic mixtures of Dns-DL-amino acids are separated by MCE in a model system using γ-CD as chiral selector (Tsai et al., 1998). However, some modifications and studies are needed to improve the racemic separation. In the present research, amino acids that are liable to racemize during alkali treatment were selected to be separated by MCE to investigate the

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effects of γ-CD on migration time, resolution (Rs) value, and Δ*t* value of the Dns-DL-amino acids. In addition, the relationships between capillary length, organic modifiers or applied voltage, and the extent of separation are also discussed herein.

MATERIALS AND METHODS

Chemicals. Dansyl chloride [5-(dimethylamino)naphthelene-1-sulfonyl chloride], boric acid, γ-CD, and DL-amino acids (phenylalanine, valine, threonine, serine, alanine, isoleucine, methionine, leucine, and glutamic acid) were purchased from Sigma (St. Louis, MO), whereas sodium dodecyl sulfate (SDS) was purchased from Bio-Rad (Richmond, CA). Sodium hydrogen carbonate, sodium hydroxide, methanol, and acetonitrile were purchased from E. Merck (Darmstadt, Germany).

Apparatus and Electrophoretic Conditions. All experiments were carried out on a capillary electrophoresis instrument P/ACE system 5500 (Beckman, Palo Alto, CA), equipped with a diode array detector monitoring a wavelength of 254 nm. An uncoated fused silica capillary (Beckman, total length = 47 cm, effective length = 40 cm, i.d. = 75 μm) was pretreated successively with 0.1 M hydrochloric acid and 0.1 M sodium hydroxide for 10 min each and then rinsed with deionized water and background electrolyte (BGE) solution prior to use. Capillary length was raised to 57 cm (effective length = 50 cm) while the effect of capillary length on enantiomeric separation was investigated. The separation column was kept at a constant temperature of 25.0 ± 0.1 °C by means of a fluorocarbon liquid continuously circulated through the cartridge, and the applied voltage was 15 kV, unless stated otherwise. Sample introduction was performed using the pressure option for 5 s. Data collection was carried out with the Gold Chromatography data system version 8.1.

The BGE composition was 150 mM SDS/60 mM γ-CD/10% acetonitrile or methanol/250 mM borate buffer solution (pH 9.5), which was filtered through a 0.45 μm membrane prior to use. Deionized water was obtained from a Milli-Q system (Millipore).

Derivatization of Amino Acids. Dansyl chloride was used to derivatize the DL-amino acids according to the method described by Nergo et al. (1987). Usually, 100 μL of 500 mM $NaHCO_3$ in deionized water and 100 μL of 20 mM dansyl chloride in acetone were added to 10−40 μg of free amino acid dissolved in 100 μL of deionized water in a screw-capped Pyrex tube. The samples were reacted in the dark for 40 min at 65 °C. Dansyl chloride solution was always freshly prepared.

The pertinent parameters, resolution (Rs), separation efficiency ($N/m \times 10^5$), and theoretical plate height (H), were all calculated in the usual manner (Wan et al., 1995; Terabe et al., 1989). Rs value was used to expressed the separation results of the enantiomers (Wan et al., 1995). Δ*t* value was calculated according to the method described by Gassmann et al. (1985) and Gozel et al. (1987).

RESULTS AND DISCUSSION

In a previous work (Tsai et al., 1998), enantiomeric separations of Dns-DL-amino acids were conducted using 200 mM SDS/75 mM γ-CD/250 mM borate buffer (pH 9.5) as BGE. However, the Rs and Δ*t* values were about 1 and 0.01, respectively, and the migration times of all of the analytes were too long (for example, migration times of Dns-D- and L-phenylalanine were 29.85 and 30.93 min, respectively.). Thus, modification was needed.

Effect of γ-CD on the Separation of Dns-DL-amino Acid. The formation of the inclusion complex between CD and analytes depends on spatial factors, hydrophobic interactions, hydrogen bonding, and solvation effects (Linder et al., 1995). Therefore, selection of an adequate inner cavity of CD is one of the key points to achieve the successful chiral separation. Armstrong et al. (1987) have reported that addition of a substituent to a complex compound could result in either increased or decreased chiral recognition, and there are at least two ways for a substituent to enhance chiral recognition by CD through increased hydrogen bonding.

Table 1. Effect of γ-CD on Migration Time (t_D, t_L), Resolution (Rs), Δ*t* Value, Separation Efficiency ($N/m \times 10^5$), and Theoretical Plate Height (H) of Dns-amino Acids[a]

Dns-DL-AA	migration time (min) t_D[b]	migration time (min) t_L[b]	Rs[c]	Δ*t*[d]	$N/m(\times 10^5)$[e] D	$N/m(\times 10^5)$[e] L	H[f] (μm) D	H[f] (μm) L
Phe	11.19	11.85	3.72	0.0573	3.348	4.205	2.990	2.378
Val	12.82	13.27	2.61	0.0345	5.072	5.275	1.972	1.896
Thr	13.41	13.65	1.23	0.0177	4.312	4.713	2.319	2.122
Ser	13.84	13.84	0.00	0.0000	−[g]	−	−	−
Ala	13.45	13.45	0.00	0.0000	−	−	−	−
Ile	13.89	14.71	4.27	0.0573	5.119	4.459	1.952	2.242
Met	14.48	15.05	2.81	0.0386	4.423	3.872	2.260	2.582
Leu	14.52	15.56	4.03	0.0691	5.633	4.988	1.775	2.005
Glu	19.84	20.29	1.43	0.0224	3.723	3.480	2.686	2.874

[a] Experimental conditions: capillary, 75 mm i.d. × 47 cm (40 cm to detector); separation solution, 150 mM SDS/60 mM γ-CD/250 mM borate buffer (pH 9.5); applied voltage, 15 kV. [b] t_D and t_L were the migration times of Dns-D- and Dns-L-enantiomer, respectively. [c] $Rs = 2(t_L - t_D)/W_L - W_D)$, where W_L and W_D were bandwidths of Dns-D- and Dns-L-enantiomers at the base line, respectively. [d] $\Delta t = 2(t_L - t_D) - (t_L + t_D)$. [e] $N/m \times 10^5$, where $N = 5.54\,(t/W_{1/2})^2$; t = migration time (min); $W_{1/2}$ = peak width at half-height of peak; m = capillary length. [f] $H = (L_d/N)$, where L_d = the capillary effective length (m). [g] −, not available.

Previously, β-CD was utilized as a chiral selector to conduct the enantiomeric separation of Dns-DL-amino acids using 150 mM SDS/75 mM γ-CD/250 mM borate buffer (pH 9.5) as BGE (Tsai et al., 1998). The Rs values of Dns-DL-amino acids, except Dns-DL-serine, Dns-DL-alanine, and Dns-DL-methionine, were slightly greater than 1.0. Therefore, replacement of β-CD by γ-CD was considered. Table 1 presents the migration times, Rs values, Δ*t* values, and separation efficiencies for the Dns-DL-amino acids separated with 150 mM SDS/60 mM γ-CD/250 mM borate buffer (pH 9.5). By replacing β-CD with γ-CD, most of the obtained Rs and Δ*t* values (Table 1) were far larger than 1.0 (for example, Rs values of isoleucine and leucine are 4.27 and 4.03, respectively) and 0.01, respectively, indicating not only that the separation of the Dns-DL-amino acid mixture reached the level of baseline separation but also that the corresponding DL-enantiomers were completely separated. In addition, the migration times of each racemic mixture were shorter than those of the corresponding mixture using β-CD as chiral selector. Thus, it is obvious that the separation condition of racemic mixture by γ-CD is much more profitable than that by β-CD. During the studies on the separation of naphthalene-2,3-dicarboxaldehyde-labeled DL-amino acids, Ueda et al. (1991) found that γ-CD is more effective due to its higher enantioselectivity with the amino acids tested. The shorter migration times, compared with those separated by β-CD, observed in this study using γ-CD as a chiral selector (Table 1) are probably due to the more stable inclusion formation between γ-CD and Dns-amino acids. Terabe et al. (1990) and Nishi et al. (1991) have reported that in the micellar solution, surfactant monomers are in equilibrium with the micelle and the hydrophobic alkyl side chains of the anionic surfactants enter and occupy the cavities of CDs, thus forming inclusion complexes, and simultaneously inhibit the interaction between analytes and CDs. γ-CD, consisting of eight D-(+)-glucopyranose units, possesses a larger

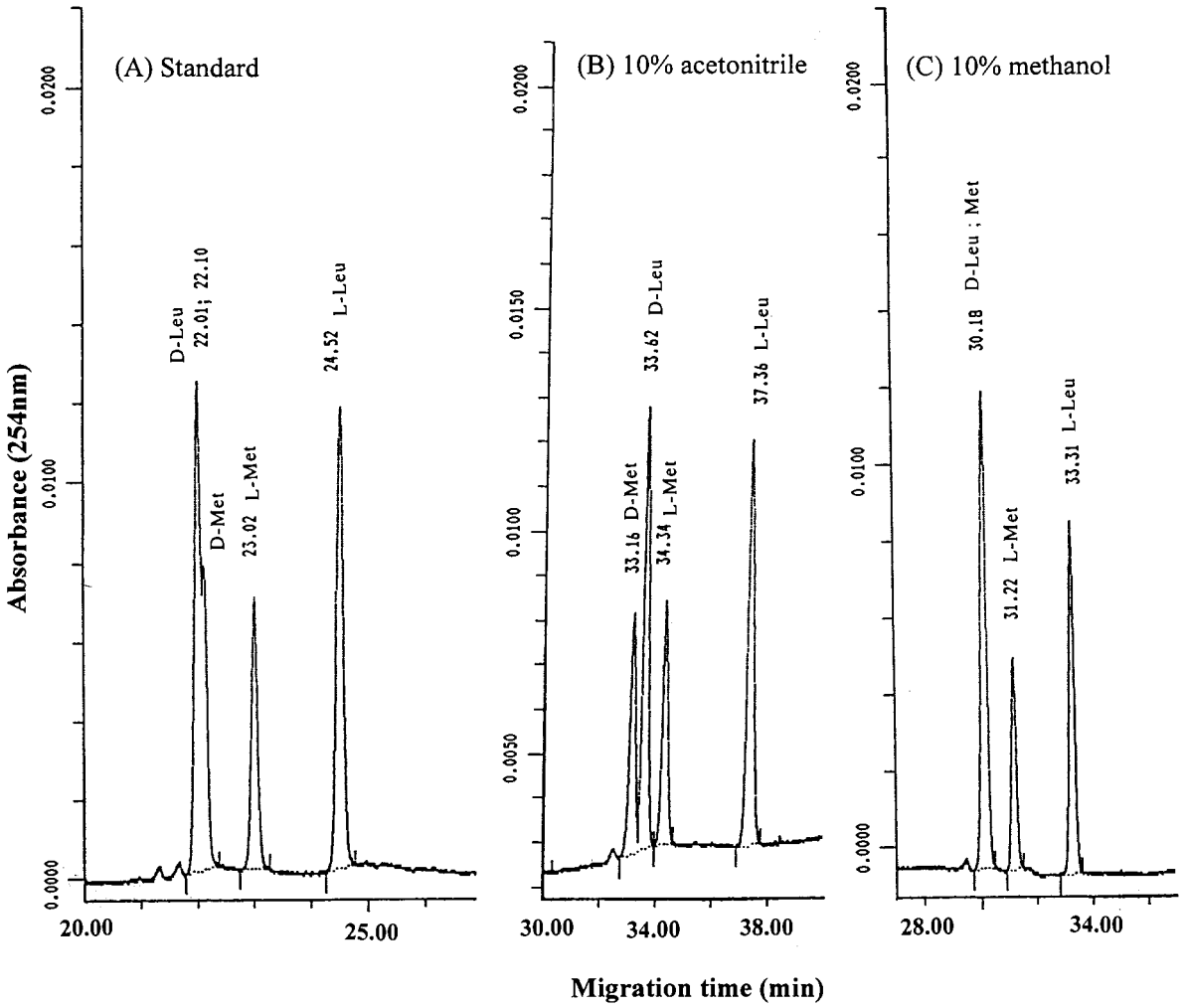

Figure 1. Effect of organic modifier addition (A, standard; B, 10% acetonitrile; C, 10% methanol) on the chiral separation of Dns-DL-methionine and Dns-DL-leucine by MEKC with γ-CD. Separation solution, 150 mM SDS/60 mM γ-CD/250 mM borate buffer (pH 9.5); capillary length, 57 cm; applied voltage, 15 kV.

cavity diameter than β-CD but still allows inclusion of the solute after the inclusion of the surfactant monomer. This leads to better resolution and shorter migration times due to the formation of more stable inclusion complex. Similar results are reported by Nishi and Matsuo (1991) for the separation of corticosteroids and aromatic hydrocarbons using γ-CD as chiral selector instead of β-CD. However, γ-CD was found to be unsuitable for separation of all the Dns-DL-amino acids that were tested in this study. During the enantiomeric separation of Dns-DL-serine and Dns-DL-alanine, the elution peaks corresponding to DL-enantiomers were completely overlapped and the migration times were the same (Table 1), probably due to their complete inclusion into the relatively large inner cavities of γ-CD, owing to their relatively low molecular weights and small molecular sizes. In other words, both Dns-D- and Dns-L-amino acids were possibly strongly included in the γ-CD cavities and could not be recognized by a chiral selector. The selection of an adequate chiral selector thus plays an important role in racemic separation, but it appears difficult to find a single selector suitable for all analytes in a single separation operation.

Capillary Length. As can be seen from Table 2, increase in capillary length enhanced the Rs values of the DL-enantiomers, because the increased capillary length allows more efficient dissipation of the Joule heat and also simultaneously minimizes the thermally induced zone broadening effect, thus benefiting the enantiomeric separation. The theoretical plate heights of all the Dns-DL-amino acids that were separated with a capillary of 57 cm in length were <2.0 μm, except only that of the Dns-DL-glutamic acid. This result reveals the remarkable effect of capillary length on the separation results. The increase in capillary length also elevated the theoretical plate numbers but was ac-

Table 2. Migration Time (t_D, t_L), Resolution (Rs), Separation Efficiency ($N/m \times 10^5$), and Theoretical Plate Height (H) of Dns-DL-amino Acids Separated by γ-CD-MCE[a]

Dns-DL-AA	migration time (min) t_D[b]	t_L[b]	Rs[c]	N/m ($\times 10^5$)[d] D	L	H[e] (μm) D	L
Phe	17.53	18.58	4.88	6.055	5.173	1.652	1.933
Val	20.53	21.26	3.32	6.312	6.468	1.584	1.547
Thr	21.03	21.40	1.49	5.431	5.867	1.842	1.705
Ser	22.09	22.09	0.00	–[f]	–	–	–
Ala	22.04	22.04	0.00	–	–	–	–
Ile	22.40	23.01	8.59	6.493	7.151	1.540	1.398
Met	22.16	23.22	3.49	6.029	5.868	1.659	1.705
Leu	22.47	25.01	4.98	5.658	5.115	1.767	1.955
Glu	30.59	31.54	1.73	3.902	3.742	2.563	2.672

[a] Separation solution: 150 mM SDS/60 mM γ-CD/250 mM borate buffer (pH 9.5); applied voltage, 15 kV; capillary length, 57 cm (50 cm to detector). Footnotes *b*, *c*, *d*, *e*, and *f* correspond to footnotes *b*, *c*, *e*, *f*, and *g*, respectively, of Table 1.

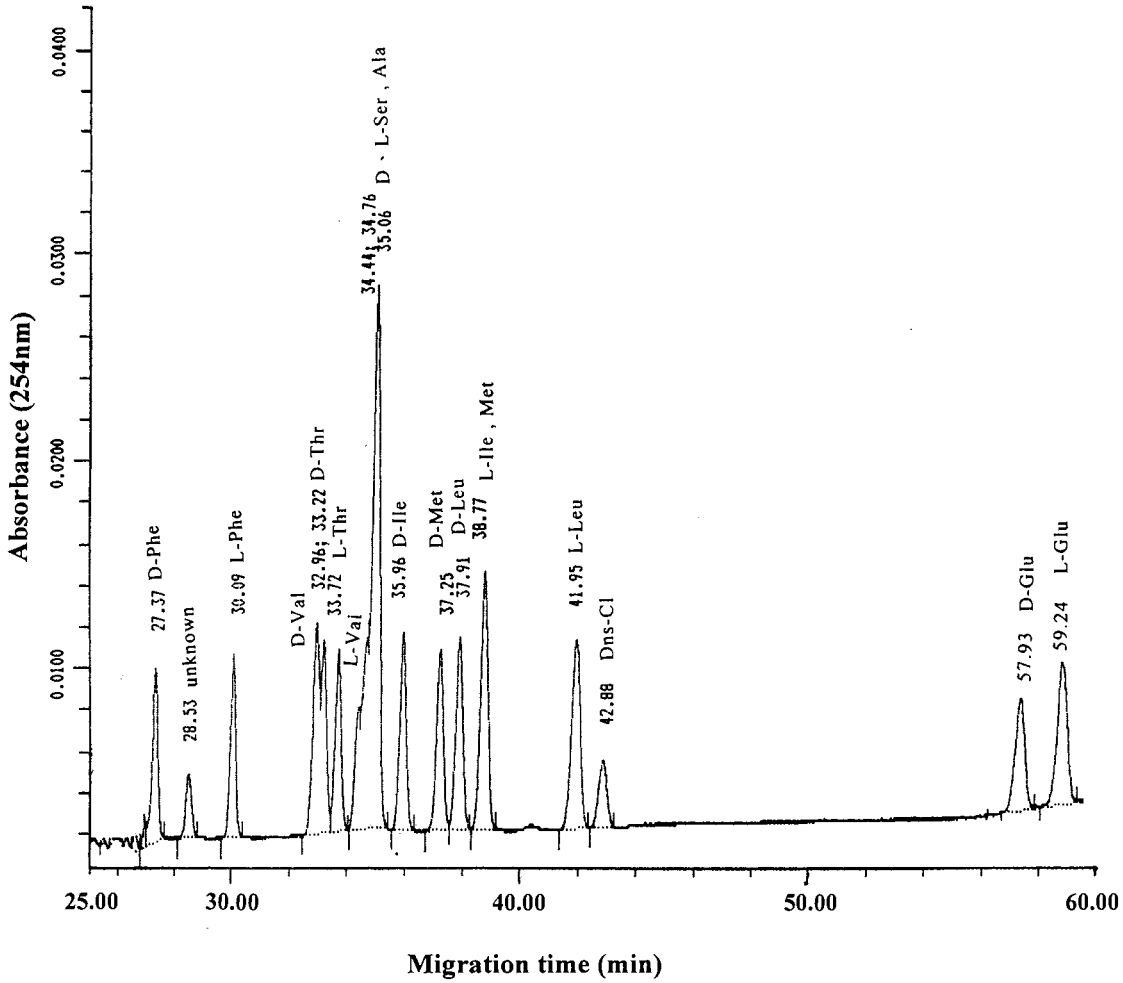

Figure 2. Effect of acetonitrile on the chiral separation of Dns-DL-amino acids. Separation solution, 150 mM SDS/60 mM γ-CD/10% acetonitrile/250 mM borate buffer (pH 9.5); capillary length, 57 cm; applied voltage, 15 kV.

companied by the drawback that migration time of the analytes was prolonged to 31min.

Peterson (1993) has investigated the effects of capillary length and inner diameters of the capillary on the separation result of (±)-epinephrine and has also reported a similar and significant result by reducing the inner diameter of the capillary and increasing its length.

Effects of Organic Modifiers on the Resolution (Rs) and Migration Time (t_D, t_L). The effects of organic modifiers on chiral resolution are closely related to the type of chiral selector and properties of the enantiomers, such as their hydrophobic and hydrophilic properties (Wang and Warner, 1995; Matchett et al., 1995). The addition of organic modifiers affects the inclusion complex formation constant and optimal concentration of CD, thus either improving or worsening the separation results. As presented previously in Table 1, γ-CD was found to be a satisfactory chiral selector for the racemic separation of Dns-DL-amino acids, but the peaks corresponding to Dns-D-methionine and Dns-D-leucine were partially overlapped (Figure 1A) and the electrophoretic condition therefore needed further improvement. For the results plotted in parts B and C of Figure 1, 10% acetonitrile and methanol were added to the BGE solution, respectively, and the separation results were compared. It can be obviously seen from Figure 1B that the addition of acetonitrile improved the separation of D-methionine and D-leucine, whereas methanol proved to be ineffective. Chan et al. (1995) have reported that addition of 10−15% acetonitrile to the buffer solution enhances the resolution of FLEC diasteromers. Wan et al. (1995) have reported improved chiral recognition of FMOC-DL-amino acids by using an additional 15% 2-propanol in the buffer. Zukowski et al. (1992, 1993) have demonstrated that, in some cases, chiral recognition could not be achieved by HPLC when the mobile phase contained water, but the separation was successful in water-free systems. They further indicated that the presence of water would force the FMOC part into the cavity of CD, thus making the interaction between amino acids and CD insufficient for enantioselectivity. However, in the water-free system with acetonitrile as the main mobile phase component, acetonitrile occupies the cavity of CD and the amino acids therefore have to interact with the external parts of CD, thus leading to the improvement of enantiomeric separation (Wan et al., 1995).

Results in Figure 1 also reveal the influence of organic modifiers on the migration time of the analytes. The increase in migration time is partly due to the reduction in the ratio of dielectric constant to viscosity, which results in the reduction in ζ potential and electroosmotic flow. In addition, inhibition of complexation with the cyclodextrin through the competition with the organic modifier for the cavity would also tend to increase migration time.

The effects of addition of organic modifiers to the buffer solution in improving the racemic separation are still unclear, probably owing to the reduction of the mobile phase polarity and changes in the partition

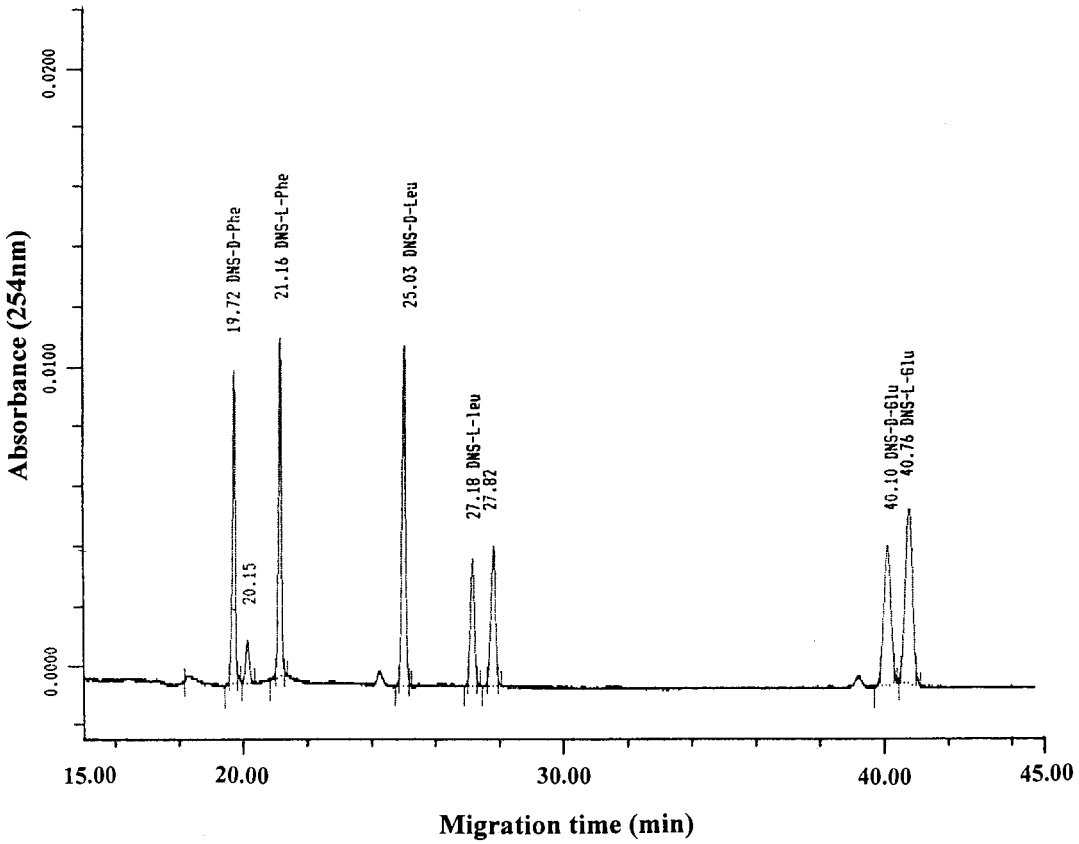

Figure 3. Enantiomeric separation of Dns-DL-phenylalanine, Dns-DL-leucine, and Dns-DL-threonine by MEKC. Separation solution, 150 mM SDS/60 mM γ-CD/10% acetonitrile/250 mM borate buffer (pH 9.5); capillary length, 57 cm; applied voltage, 17 kV.

coefficient of the analytes between micelle and mobile phase. In addition, the use of organic modifiers was effective in decreasing the affinity of the analytes to the hydrophobic cavity of the chiral selector, thus affecting the enantioselectivity and consequently improving the resolution. The interaction between organic modifier, acetonitrile, and inner surface of the capillary can reduce the electroosmotic flow and extend the migration time window, which is also expected to be effective in improving the racemic separation.

Variations in the Applied Voltage. Bjergegaard et al. (1992) have pointed out that when the capillary length is fixed, the increase in applied voltage shortens the migration time of the analytes. From Figure 2, it can be seen that the migration time of Dns-L-glutamic acid is as long as 59 min, though D-methionine and D-leucine reach the level of baseline separation. Addition of 10% acetonitrile to the BGE solution reduced the EOF and prolonged the migration time. As the applied voltage was increased from 15 to 17 kV, increase in EOF toward the cathode was larger than that in the electrophoretic mobility of the negatively charged micelles toward the anode; thus, the migration time of Dns-amino acid was accordingly shortened. The migration time of Dns-L-glutamic acid, for instance, was reduced from the original 59 to 40 min without influencing the separation results (Figure 3). The enantiomeric separation of racemic mixture of Dns-DL-phenylalanine, Dns-DL-leucine, and Dns-DL-glutamic acid were not achieved using 150 mM SDS/75 mM γ-CD/250 mM borate buffer (pH 9.5) as BGE (Tsai et al., 1998). However, using the conditions in Figure 3, separations were achieved and the separation efficiencies of these Dns-DL-amino acids were also increased. The effects of applied voltage on Rs values and theoretical plate number are presented in Table 3. The increases in Rs values and theoretical plate number are due to the increased voltage. Thus, selection of optimal voltage is expected to yield satisfactory resolution and acceptable retention time.

Table 3. Effect of Voltage on Resolution (Rs) and Separation Effeciency ($N/m \times 10^5$) of Dns-DL-phenylalanine, Dns-DL-leucine, and Dns-DL-glutamic Acid[a]

	voltage					
	15 kV			17 kV		
Dan-DL-AA	Rs	N/m ($\times 10^5$) D	N/m ($\times 10^5$) L	Rs	N/m ($\times 10^5$) D	N/m ($\times 10^5$) L
Phe	4.76	1.994	1.987	5.47	5.203	5.271
Leu	7.37	2.690	2.034	7.40	5.211	4.298
Glu	1.38	1.895	1.798	1.45	2.677	2.766

[a] Separation solution: 150 mM SDS/60 mM γ-CD/10% acetonitrile/250 mM borate buffer (pH 9.5); capillary length, 57 cm; Rs and separation efficiency were the same as in Table 1.

Conclusion. γ-CD was used as chiral selector to conduct the enantiomeric separation of Dns-DL-amino acids in the presence of SDS, and the effects of γ-CD, capillary length, organic modifiers, and applied voltage on the separation results were studied. Importantly, the result of racemic separation has been found to be dependent on the chiral recognition of CD, which is closely related to the cavity size of the CD, and the molecular weight and properties of the analytes. Enantiomer mixtures of Dns-DL-leucine, Dns-DL-phenylalanine, and Dns-DL-glutamic acid were only partially separated when 150 mM SDS/75 mM β-CD/250 mM borate buffer solution (pH 9.5) was used as BGE (Tsai et al., 1998). However, when β-CD was replaced by γ-CD, these three amino acids were completely sepa-

rated, displaying separate individual peaks. Amino acids of small molecular size, such as DL-serine and DL-alanine, could be properly and enantiomerically separated by using β-CD as a chiral selector, whereas γ-CD was found to be ineffective for this purpose. The Rs values of the Dns-DL-amino acids, except for DL-serine and DL-alanine, were larger when separated with γ-CD than with β-CD. Both migration times and separation efficiency were much more satisfactory when the racemic amino acid mixtures were separated with γ-CD. Variations in the capillary length and applied voltage improved the resolution and theoretical plate number by minimizing the thermally induced zone broadening effect. Addition of acetonitrile was found to be effective in separating D-leucine and D-methionine, through modification of the partition ratio of the analytes between the pseudo-stationary phase (SDS) and the mobile phase (borate buffer and γ-CD). Because the properties of amino acids are different and enantiomeric separation of Dns-DL-serine and Dns-DL-alanine failed in the previous (Tsai et al., 1998) and present studies, further research is therefore necessary to complete an analyzing system for enantiomeric separation of amino acids in alkali-treated proteins.

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